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RESEARCH PAPER

An integrated strategy to identify key genes in almond adventitious shoot regeneration

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Abstract

Plant genetic transformation usually depends on efficient adventitious regeneration systems. In almond (*Prunus dulcis* Mill.), regeneration of transgenic adventitious shoots was achieved but with low efficiency. Histological studies identified two main stages of organogenesis in almond explants that were induced for adventitious shoot regeneration; a dedifferentiation stage (early) and a shoot initiation stage (late). Histological observation revealed that the limitation in the recovery of transformed shoots is primarily a function of the low organogenic competence of the transformed tissues rather than transformation efficiency. To identify key genes involved in organogenesis, shoot-induced leaves and suppression-subtractive hybridization were used, to build a cDNA library from each organogenic stage. cDNA clones from both libraries were randomly picked, PCR-amplified, and arrayed on glass slides. For transcript profiling, microarray hybridization was performed using cDNA pools from both the early and the late stages. Statistically significant differential expression was found for 128 cDNA clones (58 early, and 70 late), representing 92 unique gene functions. Genes encoding proteins related to protein synthesis and processing and nitrogen and carbon metabolism were differentially expressed in the early stage, whilst genes encoding proteins involved in plant cell rescue and defence and interaction with the environment were mostly found in the late stage. The LTP/α-amylase inhibitor/trypsin gene was more strongly expressed at an early stage, as confirmed by quantitative RT-PCR, while a gibberellic acid stimulated protein gene seems to be a good marker for the late stage. These results are discussed on the basis of the putative roles of the annotated differentially regulated genes in almond organogenesis.

Key words: Adventitious shoot, almond, early stage (ES), late stage (LS), quantitative PCR, suppression subtractive hybridization (SSH), transcript profiling.

Introduction

Upon the application of appropriate exogenous signals, meristematic and differentiated plant cells in culture are sometimes able to modify their fate, behaving as true stem cells. This plasticity has given rise to the concept that plant cells are totipotent. Some of the signals perceived by the plant are so strong that they can induce a mature cell to revert, re-enter the cell cycle, and express its pluripotency resulting in the regeneration of a new whole plant. For the almond industry to profit from genetic transformation technologies, efficient and reproducible adventitious regeneration systems are essential. An efficient protocol for somatic embryogenesis has not been established for almond

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mature tissues, however, adventitious shoot regeneration has been accomplished using leaf explants of mature and juvenile *ex vitro* or *in vitro* shoots (Miguel *et al.*, 1996; Ainsley *et al.*, 2000, 2001). At present, only two genotypes can be transformed, a seedling obtained from the Portuguese 'Boa Casta' (Miguel and Oliveira, 1999; Costa *et al.*, 2006) and the North American 'Ne Plus Ultra' (Ramesh *et al.*, 2006).

With the progress in plant genetics and molecular biology (often using *Arabidopsis* mutants and tagged probes to mark events), our understanding of *in vivo* shoot meristem development and plant cell cycle regulation has progressed significantly. In shoot meristems, several genes regulating development have been identified including the *Zea mays* (maize) *KNOTTED-1* (*KN1*) (Volbrecht *et al.*, 1991), *Arabidopsis thaliana SHOOTMERISTEMLESS* (*STM*) (Long *et al.*, 1996), *WUSCHEL* (*WUS*) (Laux *et al.*, 1996), *CLAVATA1* (Clark *et al.*, 1993, 1997), *CLAVATA2* (Kayes and Clark, 1998), and *CLAVATA3* (Fletcher *et al.*, 1999). Also, the *Arabidopsis* genes *CUP-SHAPED COTYLEDON*1 and 2 (*CUC*1 and *CUC*2), have become useful markers for following *de novo* shoot development. These genes, required for the proper shoot apical meristem (SAM) formation, are expressed during post-embryonic development (Aida *et al.*, 1997; Takada *et al.*, 2001). The expression of *KN1* in maize, and its homologue in barley has been chronicled during *in vitro* axillary shoot meristem proliferation and adventitious shoot meristem (ADM) formation (Zhang *et al.*, 1998). The ADMs appeared to derive directly from *KN1*-expressing shoot meristematic cells. In *Brassica*, expression of *Brostm* (a *KN1* homologue) marked the switch from developmentally fated to shoot meristematic cells (Teo *et al.*, 2001). Cell cycle-related genes such as the *cyclin-dependent kinase type A* (*CDKA:1*) have also been extensively studied as developmental markers in *Arabidopsis* (Ferreira *et al.*, 1991) and in several other species such as *Pisum sativum* (pea) (Feiler and Jacobs, 1991), and maize (Colasanti *et al.*, 1991). The expression of *CDKA:1* was observed mainly in actively proliferating cells such as those from shoot meristems and young leaves (Colasanti *et al.*, 1991; Martinez *et al.*, 1992) leading to the suggestion that this gene is a useful molecular marker for the *in situ* identification of quiescent cells that are able to re-enter the cell cycle and follow the morphogenetic pathway (Zhang *et al.*, 1998; Teo *et al.*, 2001). It is clear from these studies that the identification of a single gene that is central to a particular developmental pathway is a critical step in understanding and following any important developmental event *in vivo* or *in vitro*. This is true even though it is also clear that a single gene is insufficient to support the initial stages of *de novo* organogenesis, which is complex and well co-ordinated.

Mutants impaired at different stages of organogenesis have also been useful in dissecting morphogenic processes. In *Arabidopsis*, temperature-sensitive mutants (*srd1*, *srd2*, *srd3*) that are defective for shoot redifferentiation have been isolated and characterized (Yasutani *et al.*, 1994; Osawa *et al.*, 1998). Kakimoto (1996) identified an *Arabidopsis* gene (histidine kinase domain-containing) *CKI1* (*cytokinin-independent1*) from a mutant tagged line, that when reintroduced and over-expressed, promotes green callus and shoot formation in the absence of exogenous cytokinin. Functional screening of an *Arabidopsis* cDNA library allowed the identification of a novel gene, *Enhancer of Shoot Regeneration* (*ESR*1), that encodes a putative transcription factor whose over-expression leads to cytokinin-independent shoot formation in root explants (Banno *et al.*, 2001). Cary *et al.* (2001) described *Arabidopsis* mutants with an increased capacity for shoot formation in tissue culture. One of these mutants established a new locus named *increased organ regeneration*1 (*ire*1). *IRE* may down-regulate the competence of vegetative tissue to respond to hormonal signals involved in shoot and root organogenesis. More recently, an *Arabidopsis* mutant named *hoc* (*High Organogenic Capacity*) was determined to have an increased capacity for *in vitro* shoot regeneration. *Hoc* root explants develop shoots *in vitro* without the exogenous application of growth regulators (Catterou *et al.*, 2002). Prakash and Kumar (2002) isolated a novel MADS box cDNA, *PkMADS1*, from a *Paulownia kawakamii* (a woody-tree species) cDNA library constructed from leaf explants induced for adventitious shoot formation. The expression of *PkMADS1* was restricted to shoot apices *in planta* and shoot-forming *in vitro* cultures, thus suggesting an essential role in shoot formation.

A genomic approach to analyse *in vitro* shoot organogenesis from roots, in *Arabidopsis*, has also been successful. Using the Affymetrix GeneChip™, which consists of a population of oligonucleotides that represent the complete genome, a gene expression profile for *in vitro* shoot morphogenesis identified the expression of *Arabidopsis Response Regulator5* (*ARR5*), type A response regulator gene. It was up-regulated by 7-fold at the time of shoot commitment, and its expression was localized to sites of presumptive shoot formation. Two hybrid His Kinases involved in cytokinin responses, *CRE1*, which encodes a cytokinin receptor, and *CKI1*, a gene that is capable of confering cytokinin-independent shoot development. Message levels of CKI1 and of the cytokinin receptor encoded by CRE/AMKA/WOL were enhanced 3-fold upon the transfer of explants to shoot induction conditions (Che *et al.*, 2002). Also in *Arabidopsis,* following the same approach, *STM*, *CUC1*, *CUC2*, and *WUS* staged expression marked the cryptic developmental events of adventitious shoot induction (Cary *et al.*, 2002).

To understand the sequence of events that take place during adventitious regeneration in almond, histology studies were performed. The work described here is not an exhaustive histology study, but rather a tissue level search for the early developmental details that are relevant to shoot recovery. Based on these studies, a cDNA microarray-based expression profiling strategy was combined with the construction of suppression subtractive hybridization (SSH) libraries (Diatchencko *et al.*, 1996), to identify differentially expressed transcripts that are specifically regulated during either the early or late stages of

organogenesis. Selected genes were characterized in real-time RT-PCR studies throughout the whole shoot induction period. Almond is a difficult species with regard to the *in vitro* regeneration of shoots and whole plants from explants, and as such this study will provide valuable genomic data for the elucidation of the steps involved in fruit tree adventitious organogenesis.

Materials and methods

Biological material

Almond micropropagated shoots were obtained from a seedling derived from the open-pollination of cv. Boa Casta and subcultured every 3 weeks on micropropagation medium as described by Miguel *et al.* (1996). For adventitious shoot induction, the first four expanded leaves of 3-week-old almond shoots were excised and sectioned across the midrib without fully separating the leaf pieces. Six to seven wounded leaves were placed with the adaxial surface facing the induction medium, in a 9 mm Petri dish containing 20–25 ml of culture medium. The explants were maintained in darkness at 24±1 °C for 21 d (Miguel *et al.*, 1996).

Agrobacterium strain EHA105 carrying the plasmid p35SGUSINT was used for transformation of leaf explants as described by Miguel and Oliveira (1999).

Histological studies

To investigate regeneration patterns, an initial study was conducted using scanning electron microscopy (SEM), which allows the preservation of large structures. This facilitates not only observations of the superficial organization of the explants but also, after sectioning, the internal organization as well. The areas inside the leaf that were identified as organogenic were also prepared for light microscopy, using either plastic or paraffin embedding for large sections (5×3×2 mm). Procedures utilized for tissue fixation and observation by SEM and light microscopy are described in the Supplementary data (Protocol S1) available at *JXB* online.

RNA isolation and preparation of suppression subtractive cDNA libraries

Leaf samples (100 mg) for RNA extraction were collected at 1, 2, 5, 8, 9, 13, 15, and 19 d during the 21 d dark incubation period for induction of shoot organogenesis. Total RNA was extracted using the RNeasy extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications. Two RNA pools were established by combining 1 μg from each of the RNA extracts from days 1 to 8 (the early stages of organogenesis) and from days 9 to 19 (the late stages of organogenesis) for construction of the SSH 'tester' or treatment cDNA populations. The 'driver' or control cDNA population was derived from RNA isolated from non-induced leaves of micropropagated shoots.

One microgram of total RNA from each experimental sample (each of the 'testers' and the 'driver') was used to synthesize cDNA using Power Script™ reverse transcriptase according to the procedure provided in the SMART™ cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The suppression subtractive cDNA libraries of the early and late stages of organogenesis were prepared using the PCR-Select™ cDNA subtraction kit (Clontech) according to manufacturer's recommendations. The 'driver' RNA was used to subtract both 'testers' (early and late stages) separately and the resultant subtracted cDNAs were ligated into pUCR19 (O'Mahony and Oliver, 1999) and cloned into DH5α competent cells. The transformed bacteria were grown on Luria Broth (LB) containing ampicillin (100 μg ml^{-1}), 5-bromo-4-chloro-3-indolyl, β-D-galactoside (x-Gal, at 40 μg ml^{-1}), and isopropyl-β-D-thiogalactopyranoside (IPTG at 0.1 mM) for blue-white screening. The transformed white colonies were randomly picked (4500 of early organogenesis colonies and 2500 of late) and grown as individual overnight cultures in LB liquid media with ampicillin (100 μg ml^{-1}) at 37 °C and 225 rpm in 96-well U-bottom plates. Glycerol (20% (v/v) final) was added for storage at –80 °C.

Construction of the cDNA microarray

Bacteria carrying cDNA clones from the early and late stage libraries were grown overnight in fresh medium until growth was confluent. Cells were sampled using a 96-well plate replicator and used directly to PCR-amplify the cDNA inserts using LacI and LacZ flanking primers of pUCR19 plasmid vector. The PCR was performed in a total of 50 μl of master mix, containing 1× *Taq* polymerase buffer, 10 mM of $MgCl_2$, 0.25 mM of each dNTP, 1 μM of LacI primer (5′-CAgTCACTg-AgCATgCgCAATCg-3′) and 1 μM of LacZ primer (5′-AgATCCTTTTTATTTTTAATTTTCTTTC-3′) and 0.75 U μl^{-1} *Taq* polymerase per 50 μl reaction mixture, in a 96-well PCR plate. The PCR conditions consisted of an initial denaturation step of 5 min at 94 °C, followed by 45 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, with a final step of 7 min at 72 °C using an MJ Research PTC tetrad-225 thermocycler (Perkin Elmer, Boston, MA, USA). The PCR amplified products were analysed by gel electrophoreses (1.2% (w/v) agarose run in 1× TAE). The PCR products were dried onto the surface of the wells by placement in a laminar flow hood, resuspended in printing buffer (3× SSC, 0.2% (wt/v) sarcosyl) to a concentration of 250 ng μg μl^{-1}, and transferred to 384-well microtitre plates. The PCR products representing 3840 anonymous clones (1920 from each library) were arrayed in duplicate blocks onto poly L-lysine-treated glass microscope slides using a GMS 417 microarrayer (Affymetrix, Santa Clara, CA, USA). The slides were processed and the cDNAs cross-linked as described by Hegde *et al.* (2000) and stored in the dark at room temperature.

Fluorescent labelling of target RNA and microarray hybridization

Total RNA (10 μg) for each target transcript population from replicate RNA samples (three per induction period) were fluorescently labelled using the two-step protocol from the 3DNA Submicro EX Expression Array Detection Kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's protocol. The labelled target was hybridized in a controlled water bath at 55 °C for 4 h in the dark.

Arrays were washed at 55 °C for 15 min in 2× SSC with 0.1% SDS, 10 min at room temperature in 2× SSC, and 10 min at room temperature in 0.5× SSC. Arrays were immediately rinsed in 100% ethanol and dried by centrifugation.

Data analysis

For the microarray data analysis, image analysis and signal quantification were performed using Imagene software v5.6 (BioDiscovery Inc., CA). Selection criteria for the elimination of outlying data point spots was as follows: (i) spot signal strength–processed signal (minus background) >3× standard deviation of the mean local background; (ii) spot signal uniformity–pixel to pixel signal variation <20%; (iii) replicate uniformity–mean processed signal (minus background) must have a coefficient of variance <0.2; (iv) replicate mean signal strength–one channel must have processed signal >1.5% of the quantifiable maximum signal. Two technical replicates (duplicate spots on the array for each cDNA) and three biological RNA replicates were used in the experiments. Data analysis was performed using GeneSpring 5.1 (Silicon Genetics, Inc.). In the *t* test a *P* value <0.05 was used to select genes with >2-fold higher expression in ES versus LS.

EST sequencing

DNA sequencing was performed by the use of a dRhodamine Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence reactions were analysed using a Perkin Elmer/ABI Prism 310 automated sequencer. The cDNA clones were sequenced using a combination of plasmid and clone specific primers: the plasmid primers were designed to match the LacZ (CGGGCTCTTCGCTATTAC) and LacI (TTCACACAGGAAACAGCTATGAC) regions of the pUC19r plasmid that are on opposite sides of the multi-cloning site. Sequences were manually edited to remove adaptor sequences and assembled via Seqman II DNA analysis software (DNASTAR Madison, WI, USA). The sequences were assessed and annotated by BLASTN and BLASTX from GenBank Database resources. Matches and hits were considered when expectation value *E* was equal or inferior to $4e^{-3}$.

Reverse transcription for qRT-PCR

For array validation, the total RNA from tissue taken for each day of adventitious shoot induction was extracted and combined in equal amounts into two main RNA populations: the early stage of organogenesis from days 1 to 8 and the late stage of organogenesis from days 9 to 20. The cDNA was generated using SuperScript II RT reagents (Invitrogen, Paisley, UK). Each RNA sample (4 μg) was combined with 1 mM of dNTP, 20 ng of random hexamers as primers, and diluted to a final volume of 12 μl with distilled water. The reaction mixture was incubated at 65 °C for 5 min and added to 5× First Strand buffer, 100 mM DTT, and 2 U μl^{-1} of RNase Out™. Following 2 min of incubation at room temperature, 10 U μl^{-1} of SuperScript II Reverse Transcriptase was added to the mixture and reverse transcription was allowed to proceed for 2 h at 42 °C. The cDNA synthesis reaction was terminated by enzyme inactivation at 70 °C for 15 min.

For the candidate gene expression studies, total RNA was obtained from the leaves at each time point during the 20 d of dark incubation for shoot organogenesis induction. cDNA was generated from 250 ng of each RNA sample using TaqMan RT reagents (Applied Biosystems) supplied by the manufacturer. The reaction mix contained 5.5 mM $MgCl_2$, 500 μM dNTPs, 2.5 μM random hexamers (primers), 1× RT buffer, 0.4 U μl^{-1} RNAse inhibitor, and 1.25 U μl^{-1} Multiscribe RT in a final volume of 50 μl. The RT reaction proceeded for 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. The cDNA concentration at the endpoint was measured by spectrophotometry. Each reaction yielded approximately 37.5 μg of cDNA.

A two-step qRT-PCR assay was employed in an effort to avoid differences in RT reaction efficiency between different genes under study resulting from possible differences in hybridization efficiencies of the 3′ specific primers (used in one-step qRT-PCR). Random hexamers were employed in an effort to ensure reproducibility and comparability between the genes and time points throughout the 20 d period of organogenesis.

Quantitative PCR reaction (qRT-PCR) array validation

Eight genes (see Supplementary Table S1 at *JXB* online) that were differentially expressed in either the early or late stages of organogenesis were selected for a qRT-PCR validation assay to confirm the expression profiles generated by the microarray analysis. The primers used in the qRT-PCR were designed using the Primer Express Software from Applied Biosystems and are detailed in Supplementary Table S1 at *JXB* online. All experiments were performed in duplicate. Quantitative PCR optimization assays were performed to ensure the specificity and maximum efficiency of the primers for the target and control sequences. Two pairs of primers were tested for each sequence. The qRT-PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using SYBR Green Technology and 96-micro-well optical plates (Applied

Biosystems). Each qRT-PCR reaction mix consisted of 1× SYBR Green PCR master mix (provided by the manufacturer), 300 nM of each primer, and 5 µl of cDNA sample, in a final volume of 50 µl. The thermal cycling profile was 10 min at 94 °C followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C. The specificity of the amplified products was verified by analysis of the dissociation curves generated by the ABI PRISM 7000 Software as well as by running the products in 4% (w/v) agarose gel in 1× TAE.

Quantitative PCR reaction (qRT-PCR) for candidate marker gene expression analysis

A total of five genes, based upon their putative identities and relevance to organogenesis, were chosen for a more detailed expression analysis to determine their possible use as markers for developmental processes. The candidate genes were: EF640654 a putative β-1,3-glucanase gene, EF640670 a putative glycine-rich protein gene, EF640686 putative proline-rich protein gene, EF640687 a putative GAST-like protein gene, and EF640716 a putative lipid transfer protein (LTP)/α-amylase inhibitor/trypsin gene. Gene-specific primers for real-time PCR were again designed using Primer Express Software (Applied Biosystems) (see Supplementary Table S2 at *JXB* online). Exhaustive qRT-PCR optimization assays were performed for each gene, to deliver the maximum possible specificity and efficiency with the selected primer pairs. The uniformity of the 18SrRNA expression levels throughout the 20 time points was confirmed before proceeding with subsequent qRT-PCR comparisons. Briefly, cDNA samples from the 20 time points from each of the three biological replicates were diluted to a concentration of 10 ng μl^{-1} and subjected to qRT-PCR analysis as described above. The obtained threshold cycle (*Ct*) values varied only slightly (less than one *Ct*) between samples, validating the use of the 18SrRNA gene as an endogenous control.

cDNA standard curves (positive controls) were generated using 2-fold dilutions of cDNA from sample nine (corresponding to the ninth day of shoot induction from one of the three replicates) ranging from 200 ng to 48 pg of total cDNA. Each qRT-PCR run included replicates of each cDNA from the adventitious shoot induction of each time point (using both the target and the 18SrRNA gene primers in separate reactions), two standard curves (one for the target gene and one for the 18SrRNA gene), and negative controls. The latter included no template controls and RNA-only controls to ensure the absence of contaminating genomic DNA.

The levels of the 18S and target gene transcripts in each sample were quantitatively assessed from the standard curves executed under the same experimental conditions (same plate) as the concomitant qRT-PCR measurement. The quantity of the target gene was divided by the quantity of control gene to obtain a normalized relative value for the target gene. The specificity of the amplified products was verified by analysis of the dissociation curves generated by the ABI7000SDS software by stepwise increase of the temperature from 60 °C to 90 °C.

Results and discussion

Histological studies

In an attempt to detail some of the developmental steps that distinguish organogenesis in regenerating almond leaves, an exploratory histological study of almond adventitious regeneration was conducted. After explanting leaves onto culture media and throughout the 20 d dark incubation period, a compact and globular callus tissue developed at the wound surfaces (Fig. 1A–G). Shoot buds emerged from the calli or from the globular structures (Fig. 1H, I) within 1–3 weeks after transfer to the shoot elongation medium under the standard photoperiod conditions of 16/8 h day/night. At day 3, no changes were visible at the surface of the leaf explants. By day 5, some cell division had occurred, particularly at the wound site, where many small and isodiametric cells with thin, irregular cell walls were observed. By day 7, explant size had greatly increased and cell proliferation was clearly observable predominantly at the midrib along the cut edges. At day 10 (Fig. 1B, F), transverse sections close to the wounds revealed groups of meristematic cells arranged in cell layers immediately under the adaxial and the abaxial surfaces, and close to the vascular tissue. These centres developed globular structures, at first still immersed in the mesophyll (Fig. 1G) and later extruding out of the leaf surface (Fig. 1C, E, H). At day 21, the leaf structure was hardly recognizable. Strong cell proliferation in the mesophyll formed a large callus close to the midrib region and near the wounded surfaces. These calli had a loose structure deep inside, but contained meristematic centres consisting of spherical masses of isodiametric meristematic cells with dense cytoplasm and large nuclei, occasionally associated with pre-existing vascular tissues. The simultaneous emergence of several shoots was observed upon rupture of the globular structure. Adventitious shoots did not develop synchronously and after 2 weeks under light conditions, several stages of shoot development could be observed.

Microscopic studies of leaf samples after cocultivation with *Agrobacterium*, under SEM and light microscopy, showed extensive bacterial proliferation, particularly near the wounds and over the proliferating cells. Bacteria were also present in large numbers inside the mesophyll in the intercellular spaces, entering through wounds and stomata and spreading to the inner tissues of the explants. Plastic-embedded leaves sectioned longitudinally through the wounded edges revealed that GUS positive cells were located mainly in the inner tissues, in the proximity of wounds, and rarely in the epidermis (Fig. 1F). The transformation assays and *gus* expression analyses revealed that the limiting step in almond transformation is not bacterial access to the inner cell layers where regeneration-competent cells are localized but it is the low regeneration ability of the transformed tissues. The 40% regeneration success of

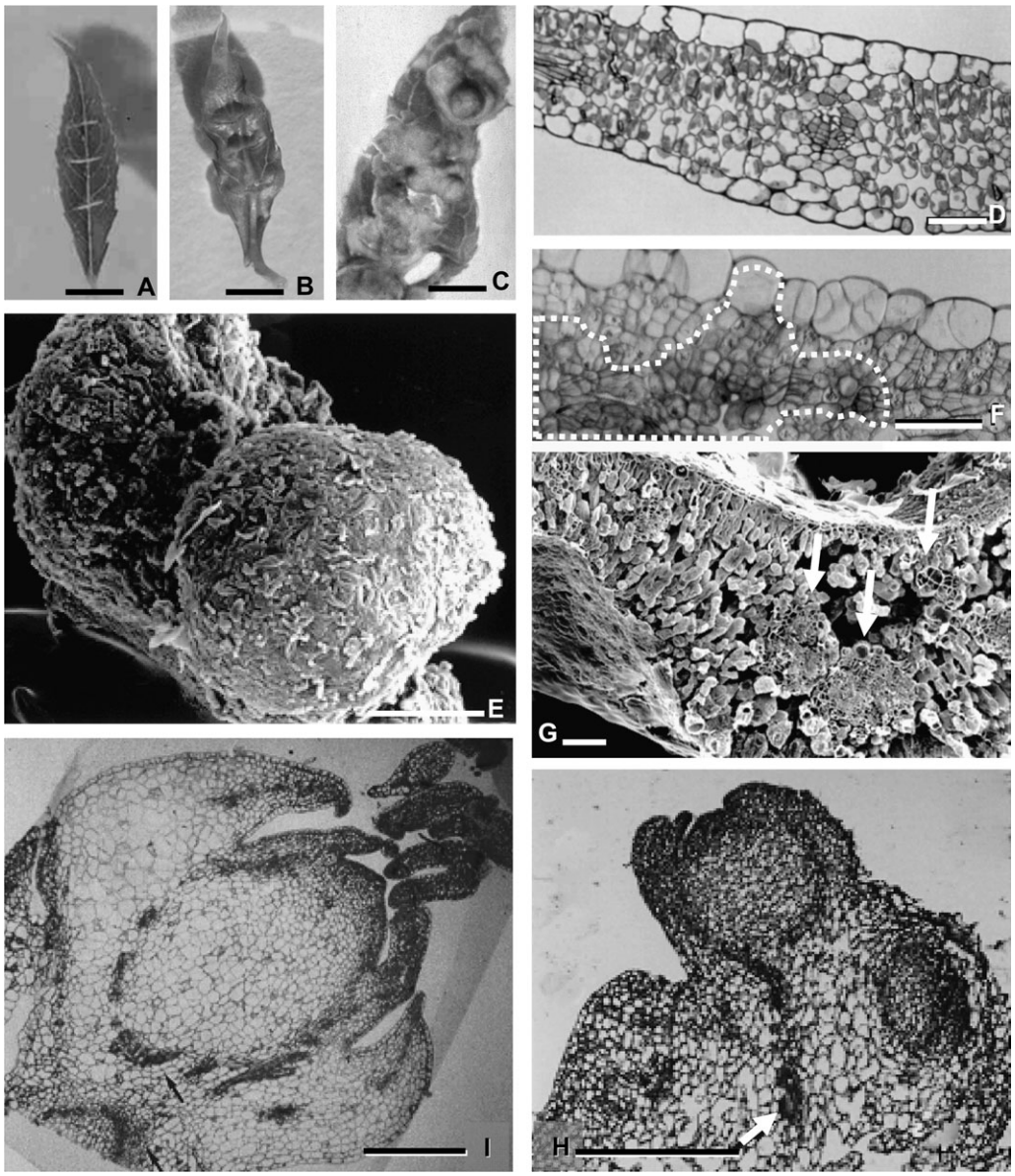

Fig. 1. Histology of the adventitious almond shoot induction along the culture period in darkness. (A, D) Aspects of non-induced leaves (day 3), (A) wounded leaf seen from the adaxial surface, (D) light microscopy image of a cross-section showing mesophyll organization). (B) Abaxial leaf surface at day 10 of induction. (C) Abaxial leaf surface at day 19 clearly showing the tissue proliferation and globular structures formed. (E) Scanning electron microscopy of globular structures formed at the adaxial surface, from which adventitious shoots arise (day 20). (F) Section across a leaf transformed with *Agrobacterium* EHA105/p35SGUSINT, at day 10 after culture initiation. Histochemical GUS expression is visible in the region delimited by a spotted line. (G) Cross-section of a leaf at day 13, showing meristematic centres at different developmental stages (arrows). (H) Two stages of emergence/development of bud initials visible at the end of shoot induction (day 20). A vascular connection is visible in the more developed bud (arrow). (I) Section across a bud emerging from a globular structure at the end of the shoot induction period (day 20). Bars (A–C) 3 mm; (D, I) 1 mm; (E, F) 50 μm; (G, H) 200 μm.

almond clone VII reported by Miguel *et al.* (1996), was found to decline with time reaching values of 25% in our experiments. This percentage reflects only the number of leaves able to regenerate at least one shoot (although, frequently, numerous shoots could be recovered from each leaf). From this situation, it is obvious that the number of cells responding to the induction signals and successfully contributing to the mRNA pool is effectively much reduced.

Although the precise histology of the meristematic centres in almond leaves could not be established, the results that were obtained allowed us to define the time frame within which the organogenesis process occurs. This knowledge was crucial in defining the frontier between the early and the late stages of organogenesis as corresponding to days 8 and 9 of almond leaf culture: almond leaves submitted to shoot induction collected at days 1, 2, 5, and 8 gave rise to an early organogenesis RNA pool and at days 9, 13, 15, and 19 represents the RNA population of the late events of organogenesis, used for SSH libraries elaboration.

Transcript profiling of almond cDNA array

To identify genes associated with the two main stages of almond adventitious shoot induction, two SSH cDNA libraries were constructed from leaves induced to regenerate. The shoot induction period was divided into two time frames, one representing the early stage of the shoot induction (ES) and the second representing the late stage (LS). The separation of these two stages was to identify genes differentially expressed (i) prior to and (ii) after commitment (or the acquisition of competency) for shoot organogenesis. A total of 3840 cDNA clones derived from two SSH cDNA libraries, 1920 from each of the early and late almond adventitious root induction libraries, were evaluated for transcript abundance and developmental relevancy in a differential hybridization expression profile analysis using a custom-made almond DNA microarray. The analysis involved a dye swap strategy with two technical replicates (duplicate spots on the array for each cDNA) and three biological RNA replicates as the source of cDNA targets for a total of six hybridizations. Of the 3840 cDNAs, 3009 exhibited measurable fluorescence (passed flags) in at least two-thirds of the hybridizations with paired probes. 616 cDNA clones exhibited fluorescent ratios that were statistically different from 1, but only 128 transcripts showed greater than 2-fold difference in abundance at $P < 0.05$. On average, only 2.8% of the array elements vary more than 2-fold in terms of reproducibility; differences higher than 2-fold between Cy3 and Cy5 fluorescence intensities in a co-hybridization experiment are believed to be significant (Ruan *et al.*, 1998). Therefore, only cDNA elements with a relative signal intensity higher than 2-fold were evaluated and those between 1- and 2-fold were not considered for sequencing and further bioinformatic analysis.

Of the 128 cDNAs that exhibit a 2-fold or greater differential expression patter, 58 cDNA clones exhibited early stage-specific over-expression compared to 70 cDNA clones over-expressed in late stage organogenesis (Fig. 2). Sequence assembly analysis revealed that these 128 differentially expressed cDNAs represented 92 unique contiguous sequences (contigs). However, 27 of the 92 contigs are close to or smaller than 100 bp in length and thus gene annotation was difficult and IDs could not be obtained. Gene annotation for the remaining 65 contigs was based on similarity to known sequences recorded in the NCBI/GenBank database, derived from BLASTN and BLASTX search protocols (Tables 1, 2). Genes encoding proteins that belong to different families were classified using the MIPS *Arabidopsis* database for protein family cataloguing (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). The functional groups within which the annotated genes clustered include various protein families; proteins involved in binding functions or cofactor requirement, cellular transport, transport facilitation and transport routes, cellular communication/signal transduction mechanism, cell rescue and defence, transcription, protein synthesis and fate, interaction with the environment, metabolism and subcellular localization (see Supplementary Fig. S1 at *JXB* online).

Of the 65 unique contigs, 37 represent transcripts that exhibited differential expression profiles that suggest accumulation and involvement in the early stage (ES) of organogenesis (Table 1) compared to 28 transcripts, which

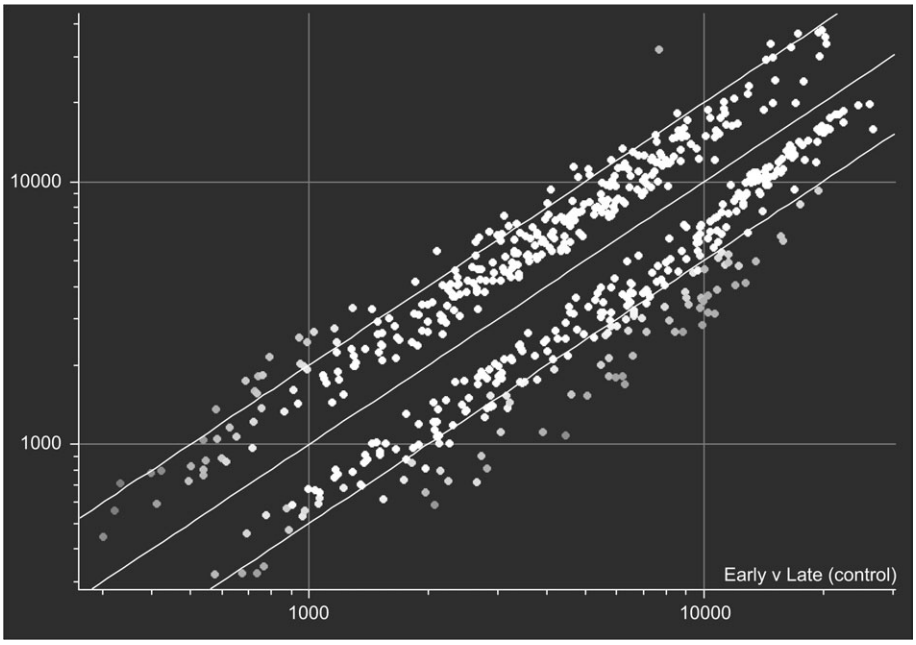

Fig. 2. Scatter plot of all genes that have a *t* test *P* value <0.05. The spots are coloured by expression ratio. The *x*-axis represents the early versus late control. The *y*-axis represents the early versus late raw data. Above the upper diagonal line are represented all the cDNA elements with an expression ratio >2-fold in the early condition versus late condition. Under the lower diagonal line are represented all the elements with an expression ratio >2-fold in the late condition versus the early condition.

Table 1. Genes differentially expressed in the early stages of organogenesis

Accession no.	Annotation	Length	Fold change
01. Metabolism			
EF640666	Putative *S*-adenosylmethionine decarboxylase (SAMD)	402 bp	2.39
EF640672	Putative ferredoxin–nitrite reductase (NiR)	554 bp	2.07
EF640674	Putative *S*-adenosylmethionine carboxypropyl transferase	684 bp	2.09
EF640684	Putative pectinoesterase	458 bp	2.14
EF640690	Putative ferredoxin–nitrite reductase (NiR)	522 bp	2.39
EF640695	Putative NAD dependent malic enzyme	720 bp	2.14
EF640698	Putative carbonic anhydrase (CA)	889 bp	2.30
11. Transcription			
EF640665	Heat shock protein HSP 90	370 bp	2.27
EF640688	Putative oligouridylate binding protein hnRNP-like protein UBP1c	737 bp	2.42
12. Protein synthesis			
EF640659	Putative purple acid phosphatase 1 (PAP1)	450 bp	2.14
EF640671	Putative 40S ribosomal protein S26 (RPS26C)	248 bp	2.04
EF640678	Putative 40S ribosomal protein S8 (RPS8A)	446 bp	2.08
EF640701	Putative ribosomal protein L36 (RPL36)	765 bp	2.00
EF640702	Putative ribosomal protein S8 (RPS 8)	602 bp	2.37
EF640704	Putative ribosomal protein L22 (RPL22)	439 bp	2.37
EF640715	Putative 60S ribosomal protein L3 (RPL3A)	876 bp	2.07
13. Protein fate (folding, modification, destination)			
EF640691	Chaperonin, 60 kDa GloEL	523 bp	2.21
EF640694	Putative ATPDILL-4 electron transporter	827 bp	2.20
16. Protein with binding function or cofactor requirement (structural or catalytic)			
EF640657	Putative calmodulin-binding/translation elongation	548 bp	2.25
EF640682	Putative translation elongation factor *EF*-1 alpha chain A4-GTP binding	322 bp	2.02
EF640685	Putative translation elongation factor *EF*-1 alpha chain A4-GTP binding	175 bp	2.14
20. Cellular transport, transport facilitation and transport routes			
EF640656	Putative non-photosyntetic ferredoxin/electron/hydrogen transport	396 bp	2.57
EF640676	Putative PQ-loop repeat family protein	313 bp	2.50
32. Cell rescue, defence and virulence			
EF640669	Putative DnaK-type molecular chaperone hsc70.1 (HSP 70)	597 bp	2.16
EF640675	Putative DnaK-type molecular chaperone hsc70.1 (HSP 70)	378 bp	2.07
EF640696	Putative polygalacturonase-inhibiting protein	397 bp	2.00
EF640716	Lipid transfer protein/α-amylase inhibitor/ trypsin	635 bp	4.06
42. Biogenesis of cellular components			
EF640686	Cell wall proline-rich protein	690 bp	2.06
70. Subcellular localization			
EF640703	Putative photosystem I assembly protein (Ycf 3)	710 bp	2.02
EF640706	Putative ATP synthase Cfi alpha subunit	640 bp	2.10
EF640709	Putative ribosomal protein L16 (Rpl 16)	320 bp	2.56
EF640711	Putative ATP-dependent protease proteolytic subunit (Clp P)	644 bp	2.07
EF640713	Putative chloroplast transmembrane protein (Ycf1)	315 bp	2.27
Unknown			
EF640663	Unknown protein	525 bp	2.26
EF640664	Untranslated leader or 3' end UTR	386 bp	2.05
EF640673	Unknown protein	473 bp	2.32
EF640681	Unknown protein	359 bp	2.60

showed late stage (LS) organogenesis over-expression (Table 2). Of the 65 organogenesis-related transcripts, 11 represent novel genes or unknown functions (4 ES-specific and 7 LS-specific).

The SSH EST collections

Gene expression profiles from two stage-specific SSH EST collections offer insight into the complex developmental events that shape adventitious shoot induction in almond. A number of cellular processes are represented within these collections and all appear to correlate well with what is currently understood about the cellular events and changes that occur as the tissues undergo dedifferentiation, redifferentiation, and organogenesis. It is clear that the two SSH collections differ in their composition. The ES collection is populated with cDNAs representing transcripts that appear to be more closely linked to an increase in metabolic activity

Table 2. Genes differentially expressed in the late stages of organogenesis

Accession no.	Annotation	Length	Fold change
01. Metabolism			
EF640693	Putative nitrite reductase (NiR)	813 bp	0.49
EF640714	Putative alcohol dehydrogenase (Adh-1)	326 bp	0.49
11. Transcription			
EF640677	Putative bromodomain-containing RNA binding protein (Brp1)	602 bp	0.45
EF640705	Putative MYB transcript factor (MYB 185)	335 bp	0.44
20. Cellular transport, transport facilitation and transport routes			
EF640670	Putative glycine rich-protein	395 bp	0.46
EF640683	Putative lipid transport for cell wall synthesis	361 bp	0.44
32. Cell rescue, defence and virulence			
EF640654	Putative β-1,3-glucanase (Gns)	426 bp	0.28
EF640658	Putative pathogenesis-related protein (PR-4A)	396 bp	0.35
EF640662	Putative acetone-cyanohydrin lyase defence-related proteins	373 bp	0.33
EF640667	Putative lipoxygenase	464 bp	0.43
EF640680	Putative disease resistance protein (TIR-NBS-LRR class)	585 bp	0.38
EF640689	Putative class IV chitinase	657 bp	0.47
EF640699	Putative peroxidase class III	484 bp	0.40
EF640707	Putative class III peroxidase ATP35	326 bp	0.24
EF640712	Putative ethylene-responsive protein (ETR)	326 bp	0.50
36. Interaction with the environment			
EF640661	Putative 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	463 bp	0.33
EF640687	Putative gibberellic acid stimulated-protein (GAST-like)	204 bp	0.48
EF640717	Putative cytochrome P450 mono-oxygenase like-T BP	186 bp	0.50
42. Biogenesis of cellular components			
EF640660	Extensin	293 bp	0.32
Unclassified			
EF640692	Metallothionein-like protein	373 bp	0.45
EF640697	Wound-responsive family protein	539 bp	0.48
Unknown			
EF640655	No hit	391 bp	0.26
EF640668	Unknown protein	636 bp	0.46
EF640679	Untranslated leader or 3' end UTR	490 bp	0.40
EF640700	Unknown protein	457 bp	0.46
EF640708	Unknown protein	130 bp	0.49
EF640710	Unknown protein	551bp	0.48
No accession	Untranslated leader or 3' end UTR	122bp	0.36

and biogenesis-related processes, whereas the LS collection appears more related to stress responses and cell wall-related processes. Perhaps this difference is reflective of the change from one cell fate to another followed by a less active period of cellular adjustment and growth.

The ES SSH collection contains a greater percentage of differentially expressed transcripts involved in protein biogenesis than seen for the LS SSH collection. These transcripts not only encode a number of ribosomal proteins but also transcripts for elongation factors (EF640682 and EF640685) that influence elongation and protein folding or transport; EF640657 a calmodulin-binding elongation factor, EF640691 chaperonin-60, and EF640694 a protein disulphide isomerase involved in oxidative protein folding in the endoplasmic reticulum (Wilkinson and Gilbert, 2004). EF640691 encodes a 60 kDa chaperonin (Cpn60) that is essential for the entry of proteins into the chloroplast through the Toc and Tic complex (Vothknecht and Soll, 2005) indicating an alteration in chloroplast morphogenesis during the initial phases of shoot induction (see below). The contribution of transcripts involved in protein synthesis and related processes are, in all likelihood, associated with cell division that occurs at about 2 d following culture initiation, apparently driven by a wounding response and the action of exogenous hormones included in the induction media (Miguel, 1998). Thus, the differential expression of genes coding for ribosomal proteins, elongation factors, and chaperonins could be a reflection of cell division of the first quiescent cells and their initial proliferation.

During the transition from the differentiated to a non-differentiated and proliferating state, in the early stages of shoot induction, chloroplasts undergo pivotal changes that ultimately lead to a reduction in photosynthesis in the cells from which new shoots arise (competent cells) (Mazari and Camm, 2005). One of the more obvious changes in chloroplast structure that occurs in competent cells during shoot induction is the chloroplastidial conversion to proplastids as the cells undergo dedifferentiation. Consistent

with these alterations in chloroplast structure, the ES SSH collection is relatively rich in cDNAS representing up-regulated plastid-associated transcripts, the majority classified in the Subcellular localization category in Table 1. In addition, EF640656 represents a non-photosynthetic ferredoxin (Fd) protein transcript associated with plastid structure and function. In citrus, Fd transcripts accumulate preferentially in tissues containing undifferentiated plastids rather than chloroplasts (Alonso *et al.*, 1995). The Fd expression patterns in citrus suggest that the induction of non-photosynthetic Fd could be related to the demand for reducing power in non-green but biosynthetically active tissues.

During *in vitro* adventitious shoot formation, the demand for nutrients by the processes involved in cell dedifferentiation and cell division becomes a priority. This demand is reflected in the differential expression of transcripts that encode nitrite reductases (NiR) in both the early and late stages of adventitious shoot induction. The LS collection, however, contains a contig that encodes a different NiR transcript than that seen in the ES collection, perhaps indicating that different NiR isoforms are active at different stages of shoot induction. Several studies have demonstrated that nitrate assimilation is an important process in regeneration (Christianson and Warnick, 1984; Selby and Harvey, 1990; Kintzos *et al.*, 2004), and that high NiR activity or expression is crucial for regeneration ability, at least for rice (Nishimura *et al.*, 2005). One would expect perhaps that nitrate reductase is also up-regulated during this process, but that remains to be determined as the EST mapping and in-depth analyses progress.

The ES SSH collection contains two ESTs, EF640666 and EF640674, that encode *S*-adenosylmethionine decarboxylase (SAMD) and *S*-adenosylmethionine 3-amino-3-carboxypropyl transferase (spermine synthase) respectively. The elevation of these two transcripts in the early stages of shoot initiation suggests the synthesis of polyamines in the explant tissue. Several studies have shown correlation between increase in polyamine levels with cell division (Bais and Ravinshankar, 2002), which could explain the up-regulation of these transcripts in the early stages of shoot initiation. Polyamines are also involved in interactions of cell wall components that contribute to cell wall rigidity and cell-to-cell adhesion (Bais and Ravinshankar, 2002) and it was recently suggested that the production of putrescine may play a specific role in wound repair, a process that is an integral part of the explant system for shoot initiation (Kuehn and Philips, 2005).

The importance of cell wall processes is also reflected in the inclusion of pectinesterase (EF640684) and a cell wall proline-rich protein (EF640686) in the ES SSH collection. Pectinoesterases have been linked to cell wall degradation during fruit ripening (Eriksson *et al.*, 2004). Proline-rich proteins represent one of five families of structural cell wall proteins that have been identified in higher plants and are temporarily up-regulated during plant development (Fowler *et al.*, 1999). Proline-rich proteins are presumed to be fixed within the cell wall in response to physical damage by a process mediated by the release of hydrogen peroxide and involving molecular cross-linking by a wall peroxidase (Cassab, 1998). Cell wall modifications appear to be more prevalent in the late stages of adventitious shoot induction if the inclusion of ESTs representing cell wall-related transcripts in the LS SSH collection is any indication. The accumulation of a class IV chitinase transcript (EF640689) suggests the cleavage of cell wall arabinogalactans during the induction process, which has been linked to signalling processes that induce cell division in embryogenic cultures (Van Hengel *et al.*, 1998). The presence of a class IV chitinase may also indicate an important role for cell wall-strengthening processes, in that chitinases coupled with an elevation in type III peroxidases (EF640699 and EF640707) suggests diferulic acid-dependent cross-linking activity that would result in strengthened cell walls (Andrews *et al.*, 2002). In addition, the ESTs encoding a glycine-rich protein (EF640670) and extensin (EF640660) are important cell wall constituents that can be highly cross-linked to other cell wall components. Glycine-rich proteins are often linked to the aromatic residues of lignin polymers (Cassab, 1998) and can be a marker for phase change in development (Gil *et al.*, 2003), which would be consistent with an adventitious shoot induction process. Extensin is a hydroxyproline-rich glycoprotein (HRGP) that is highly cross-linked to the cell wall and plays a major role in cell adhesion during plant development (Cassab, 1998) and the strengthening of the cell wall after cell division (Ito *et al.*, 1998). The LS SSH EST collection also contains a cDNA encoding a lipid transfer protein (EF640683) that has been suggested to play a role in cell wall biosynthesis in response to environmental stimuli (Kader, 1997).

Apart from the suggestion that processes involved in cell wall strengthening are important in the induction of adventitious shoots, in almond there also appears to be an emphasis on a reduction in cell wall adherence and elongation of cells. A β-1,3-glucanase (EF640654) transcript accumulates in the late stages of adventitious shoot induction and, although often attributed to be important in defence-related mechanism, the ability of the enzyme to cleave 1,3;1,6-β-D-glucans present in cell walls has led to suggestions that it is an important enzyme in several developmental processes (Akiyama *et al.*, 2004). The β-1,3-glucanase has also been described as being involved in spatial and temporal patterns in cell wall loosening that facilitate cell elongation during peach fruitlets development (Ko *et al.*, 2003) that may reflect what is occurring during the late stages of adventitious shoot induction. The almond GAST-like protein transcript (EF640687), a member of a family of genes that encode small cell wall proteins that have a cysteine-rich domain and a putative signal peptide, is also up-regulated late in organogenesis. GAST proteins are thought to play an important role in phytohormone-mediated cell expansion, as seen during flower development in *Gerbera* (Kotilainen *et al.*, 1999).

The ordered deposition of cell wall material and its composition are important factors for the co-ordination of cell division and expansion. It is hypothesized that the

differential expression of the proteins represented in the SSH EST collections described here, those that indicate the increased activity of enzymes that influence cell wall adherence properties along with those that are responsible for making stronger cell walls, are related to the development of small groups of meristematic cells within the almond proliferating callus cells that will eventually form adventitious shoots.

The latter stages of adventitious shoot induction in almond appear to be characterized by the differential transcription of genes typically expressed in mechanisms of biotic stress tolerance or interaction with the environment, more so than the early stages. This is reflected in the presence of the ESTs that encode β-1,3-glucanase, chitinase, pathogen-related protein PR-4A (EF640658), and defence-related proteins, lipoxygenase (EF640667), peroxidases, and an acetone-cianohydrin lyase (EF640662) that represent mechanisms of defence to several kinds of pathogens. It is clear that the aseptic leaf explants that are placed on the adventitious shoot induction media are not responding to a pathogen attack so it is thought that the explants are responding not just to developmental signals but also to the stressful aspects of the shoot induction protocol. In almond, it is well documented that organogenesis is only observed when leaf explants are sectioned across the midrib, which presumably means that wounding and alterations in the internal metabolite fluxes are crucial signals for the initiation of the developmental process leading to shoot formation (Miguel *et al.*, 1996). It is unlikely that wounding *per se* would be the direct cause of the induction and accumulation of biotic and abiotic stress induced transcripts during the late stages of adventitious shoot induction. However, the stressful aspects of tissue disruption as a result of mechanical tensions brought on by the growth occurring in the meristematic centres in LS, may cause the production of reactive oxygen species (ROS) and lead to the induction of the aforementioned stress response genes. Actually, it was recently documented that there is a positive correlation between ROS content in the apoplastic fluid and wall loosening in roots subjected to water stress (Zhu *et al.*, 2007). Apparently, these processes can be mediated by biotic stress proteins such as β-1,3-glucanase, chitinases, peroxidases, and several proteins with a putative N-terminal signal peptide that may lead protein targeting to the secretory pathway (Zhu *et al.*, 2007).

That ethylene may play a primary role in the later stages of adventitious shoot induction can be inferred from the increase in the accumulation of the transcript (EF640661) encoding 1-aminocyclopropane-1-carboxylate oxidase (ACCO), the enzyme responsible for the last step in ethylene biosynthesis. The regulatory role of ethylene in organogenesis has been supported by the culture of transgenic plants with impaired ethylene biosynthetic capacity, in transgenic mustard (Pua and Lee, 1985) and melon (Amor *et al.*, 1998). In these transgenic explants down-regulation of the ACC oxidase gene resulted in a decline in ethylene levels and in a marked increase of shoot regeneration. In apricot, a *Prunus* species, ethylene inhibitors were reported to improve adventitious shoot regeneration from leaf explants, suggesting that the ethylene produced by cultured explants inhibits shoot organogenesis (Burgos and Albuquerque, 2003). These studies imply that ACC oxidase activity is inhibitory to shoot morphogenesis when present in the intermediary callus. However, in *Medicago truncatula* it was shown that ethylene was necessary for somatic embryogenesis (Mantiri *et al.*, 2008). These authors have demonstrated that ethylene induces the expression of a transcription factor named *Mt Somatic embryo related factor 1*. This factor is strongly expressed in the globular somatic embryo where a high expression is found in a small group of cells in the developing shoot meristem at the heart-stage. The MtSERF1 appears to be essential for somatic embryogenesis and may enable a connection between stress and development (Mantiri *et al.*, 2008). Whether or not the increase in ACC oxidase in almond adventitious shoot induction indicates an inhibition of shoot regeneration during the late stage of organogenesis or accelerates shoot primordial formation remains to be tested.

Validation of microarray transcript profiling by quantitative RT-PCR

To confirm the reliability of the microarray data, eight differentially expressed genes were selected for expression profile analyses using qRT-PCR. cDNA was synthesized from RNA from the third biological replicate used in the array analysis along with two additional biological replicates. The criteria for gene selection in this analysis included contig length, annotation, and *E* value, and the fold-change as derived from the array analysis (Table 3). To normalize the qRT-PCR each gene was compared to the 18S ribosomal transcript, whose expression was constant throughout the organogenesis. It was possible to use this as our internal standard since the cDNA was prepared by the use of a collection of random primers rather than oligo dT. Each reaction was achieved in duplicate for each of the three biological replicates. The pattern of gene expression was conserved for all eight genes in both analyses, microarray, and the qRT-PCR, as indicated by an r^2 value of 0.95 when the data sets were compared via a regression analysis (see Supplementary Fig. S2B at *JXB* online). Although the direction of differential expression was confirmed, the ratios of the qRT-PCR were slightly different from the ones obtained in the microarray (see Supplementary Fig. S2A at *JXB* online). This is to be expected as the two methods differ in sensitivity and the biological replicates were different between each analysis.

Candidate marker genes for organogenesis

Analysis of the melting curves for all of the generated PCR products confirmed that all amplicons were single unique fragments with the expected thermokinetic properties, indicating that no non-specific products were generated in any of the reactions. The standard curves generated for all genes

under analysis presented slopes that were indicative of PCR efficiencies ranging from 91% to 100%.

The results of the candidate marker gene expression analysis for the first 20 d of organogenesis are presented in Fig. 3. Of the five candidate marker genes analysed by qRT-PCR, EF640654 a putative β-1,3-glucanase gene, EF640670 a putative glycine-rich protein gene, EF640686 putative proline-rich protein gene, EF640687 a putative GAST-like protein gene, and EF640716 a putative lipid transfer protein (LTP)/α-amylase inhibitor/trypsin gene, four seem to be promising as useful markers for stages of organogenesis in almond adventitious shoot induction. The levels of the

Table 3. Early- and late-stage specific genes selected for *q*RT-PCR validation of microarray transcript profiles

The table shows the comparison between the values obtained by both methods and the *t* test *P* value for the difference in the relative fold-change.

Accession no.	Stage	Annotation	*E* value	Length	qRT-PCR	Microarray	*P* value
EF640654	Late	β-1,3-glucanase	0.0	652 bp	0.1498	0.28	0.0018
EF640655	Late	Unknown	–	444 bp	0.128	0.26	0.0006
EF640656	Early	Non-photosynthetic ferredoxin	0.004	328 bp	1.8133	2.57	0.0869
EF640660	Late	Extensin	e^{-162}	516 bp	0.1314	0.32	0.0004
EF640661	Late	ACC oxidase	e^{-42}	554 bp	0.2729	0.33	0.0001
EF640681	Early	Unknown	$5e^{-88}$	359 bp	2.9675	2.60	0.0176
EF640675	Early	Elongation factor 1-α EF1	$2e^{-57}$	310 bp	0.929	2.14	0.2686
EF640716	Early	Lipid transfer protein/α-amylase	$3e^{-40}$	579 bp	3.7226	4.06	0.0188

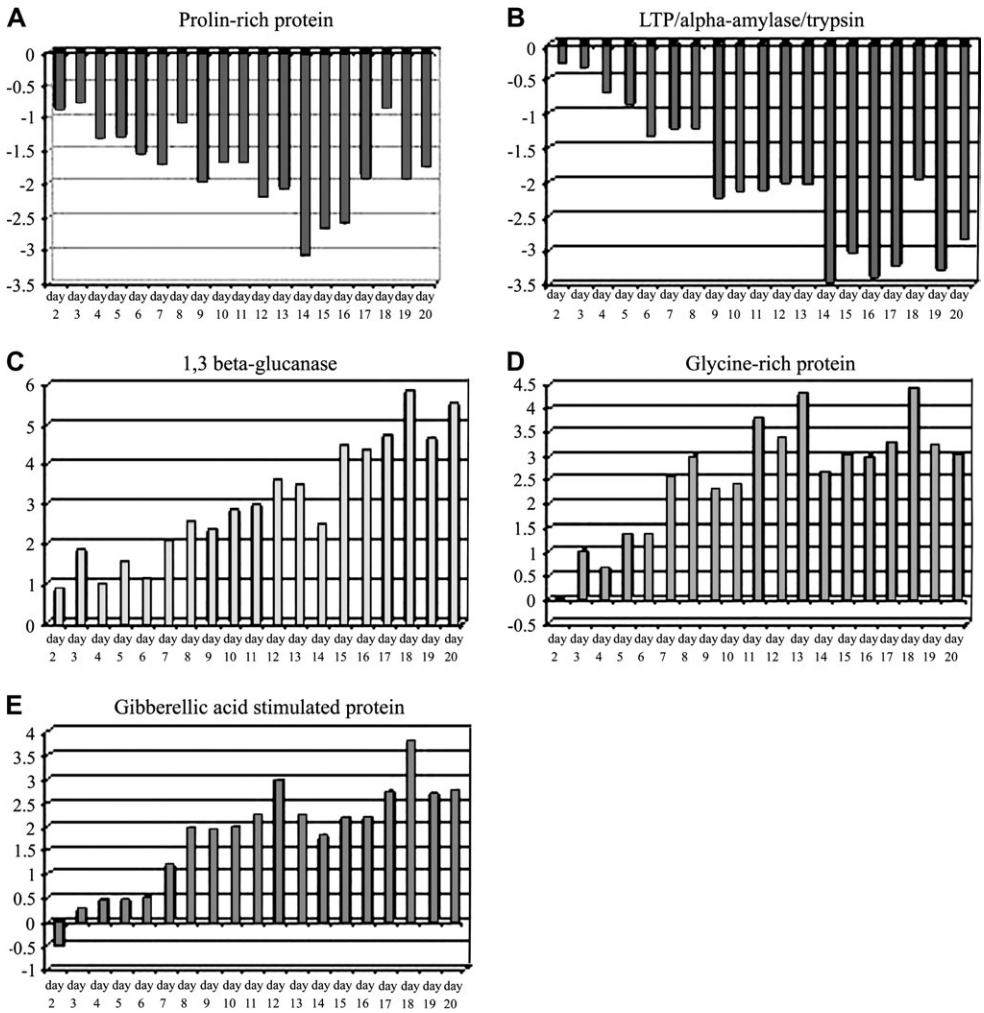

Fig. 3. Transcriptional changes of the candidate gene markers throughout the 20 d of adventitious shoot induction. The *x*-axis represents the days of the adventitious shoot induction during dark incubation. The *y*-axis represents the fold change of gene expression calculated as function of the first day using a log2 function. (A, B) Early stage candidate marker genes. (C, D, E) Late stage candidate marker genes.

transcript encoding the proline-rich protein does not have a stable enough pattern of expression to be reliable as a marker during the stages of organogenesis that take place within the 20 d time frame. The GAST-like protein gene, implicated in phytohormone-mediated cell expansion, has an elevated expression level from day seven onwards and thus marks what appears to be the transition between the early to late phases of adventitious shoot organogenesis. Indeed it appears that the period between days six and eight is a transitional one as it is during this period that the expression levels of the β-1,3-glucanase gene and the glycine-rich protein gene transcripts also start to accumulate to a higher level of accumulation, although not as abruptly as the GAST-like protein gene. The lipid transfer protein (LTP)/α-amylase inhibitor/trypsin gene accumulates more in the early stages, and declines in what appears to be programmed stages, the first of which occurs on day six. Although the true function of this gene is unknown, the encoded protein is considered to be a plant defence protein (Garcia-Olmedo *et al.*, 1998). However, it was recently associated with the pre-cotyledonary stage of zygotic embryogenesis in *Pinus pinaster* (Gonçalves *et al.*, 2005). The apparent importance of the day 6 to day 8 transition remains to be investigated, but if this is a true transition between the early and late stages of adventitious shoot induction then it may well be the time at which the explants switch from a time of high metabolic activity and cell division to cell elongation and differentiation as suggested by the composition of EST SSH collections.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Protocol S1. Experimental procedure for the histological studies.

Supplementary Table S1. Oligonucleotide primers used for qPCR array validation.

Supplementary Table S2. Oligonucleotide primers used for qPCR candidate gene marker expression analysis.

Supplementary Figure S1. Family function cataloguing of the genes differentially expressed.

Supplementary Figure S2. Comparison of expression data of eight genes using Microarray and qRT-PCR.

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